BARD

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**Manipulation Chloroplast Gene Expression: A Genetic
and Mechanistic Analysis of Processes that Control
RNA Stability**

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**BARD Final Scientific Report
Cover Page****Date of Submission of the report:** June 7, 2004**BARD Project Number:** US-3177-99C**Project Title:** Manipulating Chloroplast Gene Expression: A Genetic and Mechanistic Analysis of Processes that Control RNA Stability**Investigators****Institutions****Principal Investigator (PI):** Dr. David Stern

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Collaborating Investigators:

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Abbreviations commonly used in the report, in alphabetical order: **PNPase** – polynucleotide phosphorylase.

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Dr. Komine Y. (US) _____

Dr. Rott R. (IL) _____

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Description of Cooperation:

The collaboration has been active. In addition to publications, there have been 3 visits by co-PI Stern to Israel (1994, 1998, 1999), and 4 visits by co-PI Schuster to the U.S. (1994, 1997, 1999, 2001). These visits have stimulated contact outside our two laboratories through the presentation of seminars, and have allowed us to publicize BARD-funded work. For example, both PI's were speakers at the August 2002 Gordon Conference on Mitochondria and Chloroplasts. An Israeli student (Irena Lisitsky) spent a training period in the Stern lab, and an Israeli student (Shlomit Yehudai-Resheff) has begun postdoctoral training in the Stern lab. We have exchanged numerous strains, antibodies and gene constructs. Finally, in our opinion, the research is poised to enter a new and exciting phase, in terms of the biology, as described below.

Patent Summary (numbers) No patents

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3. ABSTRACT

New potential for engineering chloroplasts to express novel traits has stimulated research into relevant techniques and genetic processes, including plastid transformation and gene regulation. This BARD-funded research dealt with the mechanisms that influence chloroplast RNA accumulation, and thus gene expression. Previous work on cpRNA catabolism has elucidated a pathway initiated by endonucleolytic cleavage, followed by polyadenylation and exonucleolytic degradation. A major player in this process is the nucleus-encoded exoribonuclease/polymerase polynucleotide phosphorylase (PNPase). Biochemical characterization of PNPase has revealed a modular structure that controls its RNA synthesis and degradation activities, which in turn are responsive to the phosphate (P) concentration. During the funding period, new insights emerged into the molecular mechanism of RNA metabolism in the chloroplast and cyanobacteria, suggesting strategies for improving agriculturally-important plants or plants with novel introduced traits.

4. Publications (enclosed to the report)

1. Lisitsky I, Rott R, Schuster G (2001) Insertion of poly(A) sequences into an intergenic region increases transcription in *Chlamydomonas reinhardtii* chloroplasts. *Planta* 212: 851-857.
2. Yehudai-Resheff S, Hirsh M, Schuster G (2001) Polynucleotide phosphorylase (PNPase) functions both as an exonuclease and a poly(A) polymerase in spinach chloroplasts. *Mol. Cell. Biol.* 21: 5408-5416.
3. Baginsky, S., Shteiman-Kotler, A., Liveanu, V., Yehudai-Reshef, S., Bellaoui, M., Settlege, RE., Shabanowitz, J., Hunt, DF., Schuster, G. and Gruissem, W. (2001) The high-molecular-weight chloroplast RNA processing protein complex is a homo-multimer of PNPase. *RNA* 7: 1464-1465.
4. Komine Y, Kikis EA, Schuster G, Stern DB (2002) *In vivo* modulation of chloroplast RNA stability by 3' UTR homopolymeric tails in *Chlamydomonas reinhardtii* *Proc. Natl. Acad. Sci. USA* 99: 4085-4090.
5. Rott, R., Zipor, G., Portnoy, V., Liveanu, V. and Schuster, G. (2003) RNA Polyadenylation and degradation in cyanobacteria are similar to the chloroplast but different from *E. coli*. *J. Biol. Chem.* 278: 15771-15777.
6. Yehudai-Resheff, S., Portnoy, V., Yogev, S., Adir, N. and Schuster G. (2003) Domain analysis of the chloroplast polynucleotide phosphorylase reveals discrete functions in RNA degradation, polyadenylation, and sequence homology to exosome proteins. *Plant Cell.* 15: 2003-2019.
7. **Schuster, G.** (2002) The molecular mechanism of degradation and polyadenylation of RNA in the chloroplast. *Current Topics in Plant Biology* 3: 199-206.
8. Bollenbach, T., **Schuster, G.** and Stern, DB. (2004) Cooperation of endo- and exoribonucleases in chloroplast mRNA turnover. *Prog. Nucl. Acid Res.* (In press).

5. Summary of achievements

The publication list emphasizes our productivity and that the BARD-funded research, whose scope is necessarily limited, has helped to initiate other collaborations, which in turn have resulted in overall broader progress. Below we summarize the major findings of our recent BARD program.

Polyadenylation of chloroplast transcripts. Work by us and others confirmed early reports of chloroplast mRNA polyadenylation, and further showed that polyadenylation destabilized mRNA *in vitro*. To see whether polyadenylation also destabilized chloroplast mRNA *in vivo*, chimeric gene fusions with “pre-added” poly(A) tails were introduced into *Chlamydomonas* chloroplasts. These tails did destabilize transcripts, and led to genetic strategies to dissect the mechanism (publication 4).

PNPase as the polyadenylating enzyme in the chloroplast. In *E. coli*, the major polyadenylation enzyme is poly(A)-polymerase I (PAP), a member of the nucleotidyltransferase family. We found that in spinach chloroplasts, PAP activity is derived from polynucleotide phosphorylase (PNPase), which *in vitro* is responsible for both polyadenylation and the degradation of RNA (publication 2). The less discriminatory nature of PNPase, as opposed to PAP, is consistent with the poly(A)-rich [rather than pure poly(A)] tails found on spinach chloroplast transcripts. On the other hand, we found that tails added post-transcriptionally to *Chlamydomonas* chloroplast transcripts were essentially homogeneous poly(A), a property consistent with PAP rather than PNPase as a polymerase. Reverse genetic studies targeting *Chlamydomonas* PNPase, for which preliminary results are presented below, will allow us to determine whether the unicellular and multicellular systems differ in this regard.

Cyanobacterial mechanisms resemble those of chloroplasts. Analysis of cyanobacteria, believed to be related to the evolutionary ancestor of the chloroplast, revealed neither PAP activity nor a degradosome complex, but did demonstrate the existence of long heterogeneous [poly(A)-rich] tails. Therefore, the situation in at least higher plant chloroplasts and cyanobacteria may be the “ground state” for polyadenylation and degradation, with *E. coli* having evolved the degradosome and PAP (publication 5). Another interpretation is that these features were lost or gained on multiple occasions.

Reverse genetic approaches to complement biochemical analyses. The roles of PAP, PNPase and RNase E based on careful *in vitro* studies need to be verified *in vivo*. We have

begun to analyze *Chlamydomonas* and *Arabidopsis* mutants defective in PNPase, and propose to continue this line of experiments (see Preliminary Results).

Regulation of these processes. We learned that chloroplast PNPase, like the bacterial enzyme, can be regulated *in vitro* by manipulating the phosphate and nucleotide concentrations (publications 2, 6). Detailed analysis of the spinach PNPase revealed the contribution of each domain to the activity and regulation of the protein. In addition, the similarity of PNPase to the exosome complex engaged in RNA metabolism in the cytoplasm and nucleus of eukaryotic cells was established. These results suggest that the PNPase structure represents a general and evolutionarily conserved RNA-degradation machine in prokaryotes, organelles, as well as the cytoplasm and nucleus of eukaryotic cells (publication 6).

First, the collaboration has been active. In addition to publications, there have been 3 visits by co-PI Stern to Israel (1994, 1998, 1999), and 4 visits by co-PI Schuster to the U.S.

(1994, 1997, 1999, 2001). These visits have stimulated contact outside our two laboratories through the presentation of seminars, and have allowed us to publicize BARD-funded work. For example, both PI's were speakers at the August 2002 Gordon Conference on Mitochondria and Chloroplasts. An Israeli student (Irena Lisitsky) spent a training period in the Stern lab, and an Israeli student

Fig 1. The effect of P and ADP on PNPase activity. Purified PNPase (was incubated with [32 P]-RNA representing the *psbA* gene (marked by an arrowhead) for 45 min with the addition of P and ADP in the concentrations shown. Lanes C in each panel represents the input RNA. Reaction times are indicated at the bottom. Schematic representations of RNA s are presented on both sides of the Figure.

(Shlomit Yehudai-Resheff) has begun postdoctoral training in the Stern lab. under the funding of the BARD post doctorate fellowship program. We have exchanged numerous strains, antibodies and gene constructs.

6. Additional yet unpublished results

1. Modulation of spinach chloroplast PNPase activity by P and nucleotides. We assayed the effects of different concentrations of P and ADP singly or in combination, as shown in Fig 1. Increasing P strongly enhanced RNA degradation activity, as expected, since P and RNA are the substrates for degradation activity. At low P concentrations, the spinach

Fig 2. Modulation of PNPase activity for polymerization or degradation by the concentrations of P and NDPs. When degrading RNA (left side), the enzyme consumes P and produces NDPs. However, changes in their concentrations could cause the PNPase to "turn around" and polymerize the RNA, producing P and consuming NDPs.

shifted between degradation and polyadenylation by concentrations of P and ADP known to be physiologically relevant. A model is presented in Fig 2, based on the trimeric structure resolved from crystallographic analysis of the *Streptomyces antibioticus* enzyme.

2. RNase E activity is influenced by ATP and ADP. RNase E, one candidate for initiating the degradation process, displays significantly higher activity on RNAs containing 5' monophosphates rather than triphosphates. Since bacterial and chloroplast primary transcripts contain 5' triphosphates, this property of

Fig 3. Inhibition the cleavage activity of RNase E on RNA containing a 5' triphosphate. [32 P]-RNAI containing either one (upper panel), or three (lower panel) P_i groups was incubated with *Synechocystis* RNase E in the presence of ATP as shown at the top. Products were analyzed by denaturing PAGE and autoradiography. Arrows indicate the cleavage products.

RNase E, which is thought to be modulated by a phosphoryl-binding pocket, could protect these RNAs from immediate degradation. The Schuster laboratory identified an evolutionary conserved sequence, termed Box III, when the RNase E enzymes of *Arabidopsis*, *Synechocystis* and *Nephroselmis* were compared (Liveanu et al., submitted). Since Box III may correspond to the phosphoryl-binding pocket, one hypothesis is the Box III modulates cleavage activity not only by binding the 5' terminal phosphates of the RNA substrate, but also in response to the local con-

centration of free nucleotides. To explore this possibility, the *Synechocystis* enzyme was assayed for cleavage of a model substrate harboring either 5' mono- or triphosphates, in the presence of increasing concentrations of ADP or ATP. Indeed, ATP and ADP at the physio-

enzyme paused at the stem-loop structure, as does *E. coli* PNPase. However, when activity is strongly enhanced by 20 mM P, the enzyme can fully degrade the RNA (panel A). When 1 mM ADP was included, on the other hand, degradation activity was fully blocked, even in the presence of 20 mM P (panel B). When only ADP was present, a time-dependent RNA elongation was observed (panel C). These results demonstrated that PNPase activity can be

logically relevant concentrations of ≤ 1 mM inhibited cleavage of the tri-, but not monophosphorylated substrate (Fig 3). Should this hold true *in vivo*, it would be one way in which the degradative apparatus might distinguish primary transcripts as those to be protected, from degradation intermediates or damaged RNAs. Taken together with results for PNPase, it appears that local concentrations of ATP, ADP and P could potentially play an important role in controlling cpRNA abundance by regulating at least one endoribonuclease and one exoribonuclease.

3. Gene disruption mutants in Arabidopsis. A powerful method to study gene function is reverse genetics, where plant phenotype can be observed in the absence of a chosen gene

Fig 4. CSP41b, PNPase and RNaseII are targeted to chloroplasts. The constructs were introduced into tobacco seedlings by particle bombardment for the transient assay. The image shows guard cells. The chloroplast autofluorescence is observable in both cells, but only one of them has been transformed with the EYFP construct.

leles (i.e. the knockouts) exhibited poor growth on sucrose-containing medium, and were chlorotic, as shown in Fig. 5. These preliminary results suggest that PNPase activity is important for seedling devel-

Fig 5. A segregating self-cross of a heterozygous PNPase insertion was grown for 20 d on MS medium. Examples of plants genotyped to be homozygous mutant, heterozygous, and homozygous WT at this locus are shown at the same

product. We obtained a likely null allele of *PNP* from the Salk collection, and have taken it through two generations of crosses. An *RNase R/II* allele became available recently, and will be studied as part of a concurrent project.

The *PNP::T-DNA* line appears to have a single insertion, based on segregation of the selectable marker in T2 progeny, and sequencing of a *PNP*-T-DNA PCR product confirmed an insertion in exon 4 of At3g03710. That this PNPase is targeted to the chloroplast is evident since the N-terminal region targeted YFP to chloroplasts (Fig. 4). Analysis of T3 (heterozygous self-crossed) progeny revealed a Mendelian segregation of WT and mutant alleles. Interestingly, the 1/4 of seedlings pos-

cause the co-suppression line recently described [25] was apparently healthy, albeit expressing a residual level of PNPase, additional molecular and genetic confirmation is required for

our line. This is part of the experimental plan, and is currently in progress.

4. The effect of phosphate starvation on the half-lives of

Chlamydomonas cpRNAs. The completion of the *Chlamydomonas* chloroplast genome sequence in the Stern laboratory opened the door to genome-wide investigations of gene regulation. Among the experiments were measurements of cpRNA levels in response to

Fig 7. Transcription rates based on a run-on assay comparing cells grown in complete medium (+P) and those limited 48 hrs for P. Blue spots are chloroplast genes (largely within the blue oval). The trend for lower transcription rates in -P is consistent with the known metabolic slowdown.

abiotic stresses. Relevant to this proposal is the finding that P limitation caused substantial increases in the abundance of numerous transcripts within 48 hr (e.g. Fig. 6). Increases were as much as 4-5 fold, although not all genes were affected equally. To determine whether cpRNA levels were being controlled transcriptionally or post-transcriptionally under these conditions, we measured transcription rates for 96 of the genes using a pulse labeling protocol. Fig. 7 shows a scatter plot comparing transcription rates $\pm$ P, with increases in -P conditions to the left of the diagonal, and decreases to the right. Clearly, transcription rates decrease for the most part, in opposition to the RNA levels. This implicates

RNA stability, rather than transcription rate, in establishing these increased RNA levels.

5. Manipulation of cpRNA stability in *Chlamydomonas* by reducing the expression of PNPase. To determine whether reduced PNPase activity might play a role in the increased cpRNA stability induced by P limitation, we used antisense expression of a partial *Chlamydomonas Pnp* cDNA transcriptionally fused to a *ble* selectable marker cassette. Our preliminary results are shown in Figs 8-10. Fig 8 shows that the transformants accumulated antisense RNA, whereas sense *Pnp* mRNA was reduced. An antibody was developed against the *Chlamydomonas* protein, and used to confirm that the PNPase level was reduced; our data suggest about 30% remains. Next, the response of control vs. antisense lines to P limitation was explored. As expected (Fig. 9), control cells showed increases of several cpRNAs after 48 hr of P stress. However, this increase was completely absent in the antisense cells. This experiment clearly shows the importance of PNPase activity in determining how cpRNA levels are regulated under nutrient stress.

Fig. 8. The transformation construct is at the top, with cotranscription of the antisense *pnp* moiety and *ble* to avoid transgene silencing. Below, RT-PCR analysis of *pnp* sense and antisense transcripts. The products were stained with ethidium bromide (EB) or Syber Green (SG), with *fus1* as a control. The control strain (C) carried only the selectable marker cassette. Bottom, immunoblot analysis of PNPase in antisense and control strains. PNPase is marked by an arrow; this band was not detected with preimmune serum.

Fig. 9. Control or PNPase antisense cells were transferred to P-free medium, and P was restored after 48 hr (vertical arrow). RNA was isolated at the times shown across the top in hr.

Another prediction of PNPase activity reduction is that cpRNA stability might increase under non-stress conditions. To measure this, we synchronized cells using a light-dark cycle and inhibited transcription with rifampicin at 3 hr of the light cycle. As shown in Fig. 10, RNA decay was slowed, particularly for RNAs with short half-lives,

such as *tufA* (not shown) and *clpP*. Together, these data implicate PNPase as a rate-limiting enzyme in cpRNA decay, and as an enzyme which is responsive to P limitation in the growth medium.

5. RNase E and RNase II/R antibodies. As a tool for analyzing the level of the Arabidopsis RNase E and RNase II/R in mutants and under different conditions, the recombinant proteins

Fig. 10. mRNA is degraded slower in PNPase-antisense cells. Rifampicin was added at time 0, and equal volumes of cells were used for RNA analysis at the times shown (hr).

teins were produced in bacteria and antibodies were obtained. When proteins of Arabidopsis and spinach chloroplasts were analyzed by immunoblotting, a protein of about 90 kDa for RNase E and 75 kDa for RNase II/R, the predicted sizes, reacted (Fig 11).

Fig 11. Antibodies produced against the Arabidopsis RNase E and *Hfx. volcanii* RNase II/R specifically reacting with chloroplast proteins. Purified soluble chloroplast proteins of Arabidopsis (lane 3) and spinach (lane 4) were analyzed in immunoblots decorated with the corresponding antibodies. In lanes 1 and 2, the recombinant Hfx. Protein and protein extract of this organism were loaded. Only barely noticed signals were obtained when total leaf proteins were analyzed.